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THE METHOD OF ELECRON MICROSCOPY IN BIOLOGY

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The following is a summary of the work of the Laboratory of Electron Microscopy, Department of Biological Sciences, Academy of Sciences USSR.

The development of electron microscopy and application of electron physics for the purpose of creating optical equipment with higher resolution than the best light-optical microscopes immediately attracted the attention of biologists. The possibility of considerable increase of the reuolution power by the use of electrons moving at high speed opened a new era in biological investigations, the era of supermicroscopy.

Earlier, due to the limit of resolution of the light microscope at 0.24 and of the ultraviolet microscope at 0.1 μ, many fine details of cell structure, their vitally important structures, and also the whole world of ultramicroscopic creatures and matter which invade the living organism, are the causative agents of many animal and plant diseases and even affect bacteria, namely, the world of the so-called animal viruses, plant viruses, and bacteriphages, all these remained inaccessible to observation. Starting with the creation of the electron microscope, new perspectives were opened to biologists for the study of the cytology of animal and plant cells and microorganisms, while a new chapter opened up in the field of virusology, the morphology of viruses.

The striking progress of microscopy technique, connected with the appearance of the electon microscope brought about the discovery of a multitude of factors which still do not fit within the framework of existing views and hypotheses and make their re-examination necessary. The pictures appearing the screen of the electron microscope before the eyes of the biologist cannot in many cases be explained on the basis of the data of light-optical cytology and histology. The necessity of quickly overcoming the Leeuwenhoek period in the development of the

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electron microscope required systematic and planned investigations on disclosing the nature of the observed pictures; there arose the pressing necessity for working out methods which would prevent the appearance of all kinds of artifacts on the screen of the electron microscope and which would expand the possibility of using the electron-microscope method in the interests of biology.

These tasks were presented by the Presidium of the Academy of Sciences USSR to the Chamber of Electron Microscopy of the Department of Biological Sciences. This chamber was organized 4 years ago by request of the Bureau of the Department of Biological Sciences. It was founded as an inter-institute body, to work in contact with the biological institutes and laboratories of the Academy of Sciences USSR, which were also interested in the use of an electron-microscope method.

It should be noted that the chamber, soon reorganized into the Laboratory of Electron Microscopy within the Department of Biological Sciences of the Academy of Sciences USSR, was one of the pioneers in our country in the field of applying the electron-microscope method to solving problems of biology. Together with the Laboratory of Biophysics of the Institute of Experimental Medicine of the Academy of Medical Sciences USSR, the Laboratory of Electron Microscopy became a sort of school for the nuclei of biologists rising up in the scientific institutes of the USSR, who were aiming at using the electron microcope in their investigations. Shortly after the organization of the Laboratory of Electron Microscopy, specialists in various fields of biology, who had acquainted themselves with the investigations carried out there, began visiting it, and studied its work, particularly the laboratory's method for preparing biological specimens for electron microscopy.

Useful consulting aid in connection with organizing the work with the electron microscope was given by the laboratory to the institutes of the Ural Affiliate of the Academy of Sciences USSR, the Academy of Sciences Latvian SSR, the Academy of Sciences Belorussian SSR, the Moscow and Leningrad Institutes of the Academy of Medical Sciences USSR, and to a number of other scientific research institutes of the country.

The Laboratory of Electron Microscopy is now the leading scientific institute in the field of application of electron microscopy for biological investigations, judging by the scope and volume of its investigations, by the results of the applied method of electron microscopy, and by the character of the scientific-methodological assistance given to other scientific research organizations.

The inadequacies of the existing methods of cleaning biological specimen for electron microscopy came to the foreground during the first steps of the laboratory's investigative work. As is known, cleaning of biological objects, particularly microbiological and virusological ones, from impurities (components of the medium and products of cell metabolism and decomposition) is an essential condition of electron-microscopy investigations, since in drying of the specimen, these impurities may obscure the object itself.

In a number of German and American works, the method of washing the specimen with distilled water by centrifuging was used for this purpose. However, the centrifuging process can give rise to various morphological and cytological changes of the object: disturbances in the plasma, injury to the flagella in bacteria, falling apart of cell chains, artificial disconnecting of various cell elements in incipient lysis, etc. The American method of washing bacteriological specimens, the so-called meniscus method, is not free of shortcomings, either. In this method, the greater protion of the cells is washed by a meniscus together with the substances of the medium, and the experimenter cannot be certain that the normal configuration of the cells has been maintained.

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The laboratory was confronted with the task of working out a new method which would permit cleaning the object without damaging it, and at the same time create favorable conditions for the continuous study of ontogenesis of cells or various physical or chemical effects on the cell, as revealed on the film of the "object slide" of the electron microscope under application, of the suspended drop or agar-agar chamber procedure. Such a method was developed. Its principle consists of the use of a supporting colloid film, which is used for preparing an "object slide" effective as a dialyzing diaphragm. This method, called the drop dialysis method, not only guarantees a sufficient degree of purity of the biological specimen, but is also of great value in the electron-optical study of various stages of the individual development of cells. The method further facilitates the electron-microscopic investigation of change occurring in the cells under the action of physical, chemical, or biological agents, and permits tracing the dynamics of the action of various factors on biological objects, as the experimenter can easily vary the concentration or exposition of the object to the action of the required factor while the object, in the drop dialysis method, is inclosed in the drop medium on the "object slide."

A number of projects in microbiology, virusology, and biochemistry were carried out at the laboratory with the aid of the drop dialysis method.

One of the basic investigations conducted by the laboratory was the study of the cytomorphological changes of bacterial cells under the action of various agents, including antibiotics. It is known that the group of compounds with the capacity of causing bacteriolysis -- decomposition of the bacterial cell -- is extremely large and getting still larger as the investigation of the field of antibiotics develops. In this group, there are the most varied substances, produced by animal and plant organisms as well as by microorganisms. To the bacteriolytic agents belong the bacteriophages and such substances as formalin. Among them one can encounter on one hand substances of comparatively simple chemical composition, and, on the other, high-molecular protein compounds whose composition has not yet been clarified.

The phenomenon of bacteriolysis is extremely widespread, and there seem to be no microorganisms which will not be affected by some bacteriolytic agent. However, under the light microscope it is difficult to detect any peculiarities of the disintegration of the bacterial cell which are connected with the species of the microbe or with the nature of the lytic agent. Therefore, there is great interest in the question as to whether some specific features can be traced in bacteriolysis with the aid of the electron microscope or whether new details in the process of bactericlysis can be discovered if the pictures of cell decomposition of the same bacteria, caused by different agents, are compared with each other.

All investigations were carried out with sporiferous bacteria isolated from the soil, which were sensitive to the section of antibiotic substances, bacteriophages, and other agents. At first, electron-microscope pictures of the stages of the development of these bacteria, not complicated by exerting any influence on this development, were examined. It was necessary to establish a clear idea of the age changes of the bacteria, starting within the first hours of cultivation and ending with the changes after the death of the bacteria.

After the electron-microscope investigations had been carried out, it became obvious that with the interruption of cell multiplication the age changes in the composition of the colloid of the protoplasm are different, depending on whether the development of bacterial cells leads to formation of spores or whether no spore formation takes place. In the first case, part of the plasmatic content is retained and constitutes the spore substance, while the remaining elements of the protoplasm disintegrate. Electron-optically, this process /of partial disintegration does not differ from the picture of the disintegration of the total colloids of cells in which no spores are formed./

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In both cases, the process proceeds along the line of disintegration of plasmatic elements, i.e., reduction of their density and mass as a consequence of the breaking up of high-molecular compounds into low-molecular ones. On the screen of the electron microscope, this process is expressed by the gradual increase in the electron permeability of various parts of the protoplasm, and then of the entire cell, which becomes entirely transparent, "becomes empty," until finally only its membrane remains visible. In cells which did not form spores, the membrane does not collapse for a long time, which allows us to conclude that the "empty" bacterial cell is full of liquid which is permeable to electrons and has a pressure equal to that of the medium.

If this sporiferous bacterium is subjected for a short time to the action of such antiseptic substances as chloroform, 96-percent ethyl alcohol or toluene, the same picture of protoplasm breakup, of gradual reduction in size and dissolving of individual parts, and also of almost completed cells which cleared up and in which only small parts weakly absorb electrons, can be seen in the cells killed by the antiseptics. In these cases, too, the processes of hydrolytic decomposition of the protoplasm components do not touch the cell membrane. It is not destroyed and is visible in the "empty" cells as a transparent shell which retains the shape of the cell.

At the basis of this electron-microscope picture of the disintegration of the contents of bacterial cells lies the action of the cell's own enzymes. It is sufficient to heat a suspension of bacteria to 80°C for half an hour, in order that, after temperature inactivation of the cell enzymes has been accomplished by this heating, the picture of protoplasm disintegration will no longer be observed. Consequently, the process of destruction of cell components in dead cells (among them those killed by the brief action of such substances as alcohol, chloroform, or toluene) can be characterized as a process of autolysis, taking place without destruction of the integrity of the membrane and thus creating the impression of complete "emptying" of the bacterial cell.

However, such a type of posthumous cytological change in bacterial cells is not unique. In electron microscope investigation of the reaction of sporiferous bacteria to different doses of gramicidin, one can easily convince oneself of the fact that the process of the destruction of cells of these bacteria can take place also in other visible forms. For instance, after 6 hours of action of gramicidin in a concentration of 1 gamma per ml on cells, the protoplasm of which disintegrates inside the uninjured membrane, individual cells are encountered whose membrane has been damaged. As the exposure to the action of gramidicin increases, the number of cells with destroyed numbrane increases noticeably. This type of cytomorphological cell change, the peculiarity of which is the destruction of the membrane, can be characterized as lysis.

In experiments with gramicidin it became obvious that in the action of this antibiotic substance, even in considerable concentrations, but not so high that they suppress the hydrolytic action of the bacterial enzymes, the process of destruction of the bacterial cell takes place for the first hours without the destruction of the integrity of the membrane, i.e., it is of the autolytic type. Only with sufficiently prolonged action of gramcidin does this process develop, in a number of cells, into their complete destruction as a consequence of the disintegration of the membranes, i.e., partial lysis of the bacterial cells sets in.

The lysis of bacteria under the action of gramicidin is their autolysis, but one which takes place with destruction of the membrane. It is called autolysis, since the process of destruction of the cell components is carried out by the enzymes of the cell itself. This is easy to verify if cell enzymes are subjected to chemical inactivation using a very large quantity of gramicidin. It can be

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established by electron-microscopic investigation that the cells killed by such doses of gramicidin will not autolyze. In them, a process of sudden shrinking of the plasmatic contents takes place, which leads to a considerable reduction in volume and to thickening of the protoplasm. Such a type of posthumous cytological change in bacteria cells is characterized as coagulation.

While the processes of cell destruction taking place within the preserved membrane (i.e., those of the autolytic type) or accompanied by destruction of the integrity of that membrane (i.e., of the bacteriolytic type) express the direction and the activity of the enzymatic reactions in the cell after its death coagulation of the plasmatic content indicates complete paralysis of the enzymatic apparatus of the cell.

It seems as though (comparing the electron-optical pictures of autolysis of bacteria in old cultures and the pictures of cell autolysis in young cultures killed by chloroform, toluene, alcohol, or gramicidin) that the differences in the reactions, conditioned by the nature of the bactericidal agent and leading to the death of the cell, disappear after the death of the cell and level out into reactions of cell destruction. The impression is created of complete identity of posthumous enzymatic reactions in the cell regardless of whether its death occurred after completion of its individual development, or whether it was killed by chloroform, toluene, alcohol, or gramicidin.

However, such ideas are easily dispelled in comparative analysis of the investigations which were carried out. The specificity of the factor which caused the death of the bactera cell can be traced even in the posthumous phenomena which take place in the cell. This specificity can be evaluated on the basis of the different rates of the process of cell autolysis. This autolysis apparently depends on the manner in which the enzymatic hydrolysis reaction of the components of the bacterial protoplasm has been activated by the given bactericidal agent. On the basis of the experiments performed, the following series can be set up according to the time of appearance of autolysis in the main quantity of cells of sporiferous bacteria. In cultures of those bacteria which have gone through the development cycle without having been subjected to any special action, the main quantity of cells reaches its final stage of intracellular disintegration only on the fifth or sixth day; in cultures killed with alcohol, within 9 hours; while in cultures killed with gramicidin, the majority of bacterial cells exhibits autolysis after 1½ hours.

Here we encounter a phenomenon which considerably amplifies our ideas about the activators of enzymatic processes. In addition to the activation of individual enzymes or of a definite enzymatic system, agents must be present which are capable of simultaneously activating the hydrolytic action of many cell enzymes which belong to most varied classes, which is externally expressed by the quickly proceeding decomposition reaction of the substrates which vary as far as chemical composition is concerned and which make up the body of the bacterial cell. As yet, it is difficult to understand the mechanism of this phenomena. Whether the substance which accelerates the cell autolysis process has universal action in regard to the hydrolytic activity of the various cell enzymes, or whether it activates the "trigger" process which directs the work of the enzymes toward hydrolysis directly or by removing the action of the inhibitor, those are questions which must be left to further investigation. However, the character of the process itself permits the conclusion that a number of substances are capable of activating the enzymatic reation of autolysis in the bacterial cell and can consequently be called autolysis catalysts.

Interesting investigations were carried out at the Electron Microscopy Laboratory on the connection between the structure of plastids in cells of higher plants and the activity of the cell enzymes. It was known, on the basis of biochemical data, that the main quantity of plastid enzymes is present in a

firmly absorbed, hydrolytically inactive state and begins to exhibit its activity only after prolonged autolysis of the plant cells. It was important to clarify the character of the structural change of the plastids as a result of their autolysis and of the interdependence between the stages of disintegration of the cell components and the activity of the enzymes contained in them.

Electron-optical observations were carried out parallel to the above on the cytological changes of plastids in the process of their autolysis. Biochemical determination of the activity of enzymes in various stages of destruction of the cells, in combination with these observations made it possible to clarify with what changes in the structure of the leukoplast of the plant cell the increase of activity of the cell enzymes is connected, and to obtain confirmation of the fact that the cell enzymes are tied up with definite structures of the cell, the destruction of which causes their desorption.

The use of some solvents gives rise to the idea that the absorption of cell enzymes takes place precisely on those surfaces of the cell structure which are composed of lipoid substances.

The electron microscope permits observation of the difference in structure of plant cell plastids depending on the presence of chlorophyll. In white plastids the granules which are well visible in those containing chlorophyll are not always detected; in green plastids these granules have different dimensions. White tissue is also distinguished from green tissue by the presence of a large quantity of ultramicroscopic crystals which are a unique electron-microscopic diagnosis of the fact that metabolism in white tissue is different.

The laboratory also conducted work on the study of the flagellate apparatus of bacteria. According to contemporary data, the diameter of the flagellae does not exceed 30-35 mμ, and thus, naturally, the electron microscope allows us to see much that is new and of interest in the structure of the bacteria's locomotive organ. Up to the time at which the electron microscope came into use, some researchers denied even the existence of the flagellae, and considered them artifacts arising due to manipulation of the cells.

The structure of the flagellate apparatus was studied slightly on free soil bacteria which assimilate gaseous nitrogen -- the so-called B-azobacter -- which were used as an object of electron-microscopic investigation. Eleven azobacter cultures from various soils of the USSR were subjected to electron-microsopic investigation. It turned out that all cells of these cultures have flagellae which grow out from the entire surface of the bacterium, but which differ greatly in quantity and in character of structure in different species and even in different individuals belonging to the same species.

These observations not only have restricted importance as characterization of a detail of the morphology of bacteria, but they are also of much wider biological interest, showing that the similarity in the external appearance of the azobacter, regardless of the geographic zone and the composition of the soil in which it lives, is only apparent. More detailed study with the use of the electron microscope reveals the existence of differences in the structure of the azobacter cells, determined by the medium of their existence. It has already become clear that the structure of the flagella of the azobacter should be a systematic indication for its classification.

A number of projects was carried out by the Electron Microscopy Laboratory in the field of virusology. An attempt was made to find in plant cells the "stolbur" virus, the causative agent of one of the virus diseases of plants. It is not impossible that the fine thread-like formations which look like tobacco-mosaic virus and which are frequently encountered in cells of plants infected by the stolbur virus are in fact this virus. However, further electron-microscopic investigation is required for a definite clarification of this question.

With the aid of the electron microscope, a species of bacteriophage which acts on lactic acid bacteria was investigated and its characteristics established. A number of observations were also conducted on the character of the attachment of the bacteriophage to live and dead cells of the yellow staphylococcus [staph. citreus?] and B. coli. These observations are of interest for clarifying the role of the "tail" of bacteriophages in the phenomena of its penetration into the bacterial cell or its absorption by the cell.

The above is a breif outline of the results of the activity of the Laboratory of Electron Microscopy. This is only a beginning of broader investigations contemplated for solving fundamental questions of contemporary biology.

Work is contemplated on such important problems as the nature of microorganisms and of noncellular forms of life. The investigations on the character of the cytomorphological changes of bacteria cells under the influence of various physical and chemical agents will be continued, with the purpose of clarifying the specificity of the delicate reactions of the cell to external influences. It is planned to develop investigations for clarifying the effect of various factors on the state of the protoplasmic structure in plant cells in connection with the activity of its enzymatic system. Parallel electron-optical and biochemical investigations may be expected to establish the connection between definite enzymes and individual structures in plant cells. The peculiarities of the structure of microorganisms in connection with their significance for setting up of an evolutionary system of microscopic beings will be studied.

It is also planned to develop work on problems of bacteriophagy and virusology. The form and size of various bacteriophages will be studied, and also the dimensions of some plant viruses. These comparative investigations are directed toward explaining the features distinguishing individual bacteriophages and viruses, particularly toward the establishment of a connection between the changes of form, as well as dimensions of phages and virus particles and their activity.

Undoubtedly, in the present phase of development of the method of electron microscopy, the possibilities of its use in biological science are still far from exhausted. It is therefore essential that biological investigations based on the utilization of this fruitful method be intensified.

There is a necessity for coordinating the efforts of physicists and technicians for the further development of electron microscopy, for increasing the resolving and "probing" capacity of electron rays, and for facilitating the use of the electron microscope in biological investigations. Close cooperation between biologists on the one hand and physicists and technicians on the other will undoubtedly contribute to fulfillment of specific requirements set up by biologists for electron microscopy, and will consequently stimulate further introduction of this method into the biological and medical sciences.

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